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Restoration of Normal Hox Code and Branchial Arch Morphogenesis after Extensive Deletion of Hindbrain Neural Crest

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Among the derivatives of the cephalic neural crest is the ectomesenchyme which subsequently constitutes most of the craniofacial skeleton. There is evidence to suggest that the skeletogenic fate of the hindbrain neural crest is specified before emigration from the neural tube and that Antennapedia class Hox genes are involved in that process. To explore the putative causal link between Hox expression and craniofacial morphology, we produced a specific series of bilateral crest deletions in chick embryos and assessed branchial arch morphology, Hox gene expression, and patterning of skeletal structures in the postoperative embryo. Surprisingly, we found that deletion of the bulk of the rhombencephalic crest and substantial portions of the dorsal rhombencephalon did not prevent normal branchial arch morphogenesis and normal patterns of Hox gene (-*A3* and -*B4*) expression 48 h after operation. Neural crest-like cells have been identified on crest migration pathways at the level of the original ablation, further confirming that ablated cephalic neural crest is replaced by regeneration from the cut edge of the neuroepithelium. Furthermore, in such embryos ectomesenchyme from regenerated crest is able to form a facial skeleton in which the mandible and hyoid apparatus are normal in size and organization. These findings demonstrate that the cranial neuroepithelium has more extensive regenerative capacities than was previously thought, which has important implications for investigations of craniofacial development.

INTRODUCTION

The development of the vertebrate head involves a cascade of interactions between tissues of different embryonic origins. A key role is played by the cephalic neural crest, which gives rise to much of the connective tissues of the face and skull vault, components of a series of ganglia associated with the hindbrain, and a range of glandular and skeletal structures in the neck (Le Douarin, 1983; Couly *et al.*, 1993). The rhombencephalic neural crest is of particular interest in the development of the head, as there is some evidence that it contains spatial information which controls the form of the skeletal elements that arise from it (reviewed in Hanken and Thorogood, 1993; Thorogood, 1993). This was shown in experiments in which the first arch crest from quail embryos was used to replace the second arch crest of chick hosts before crest migration had occurred (Noden, 1983). The first arch crest migrated into the second arch of the host embryo, but there gave rise to elements of the first arch skeleton. Furthermore, the neural crest seemed able to alter the spatial patterning of other tissues with which it came into contact, as the duplicated skeletal elements were associated with a set of muscles of first arch phenotype but derived from the paraxial mesoderm of the second arch. In addition to alterations in mesoderm patterning, the ectoderm was also affected, in that an ectopic beak derived from second arch tissues was present. The latter findings have led to the suggestion that an intrinsic spatial specification within the forming neural tube influences the development of the other tissues in the head via the neural crest, which is itself able to transfer axial positional information to those other tissues (Noden, 1983; Hunt *et al.*, 1991c).

Recent work with vertebrate Hox genes has raised the possibility that this region-specific pattern specification is the result of the combination of Hox genes expressed by the crest population derived from a particular axial level. Different axial levels of the hindbrain neural plate express particular combinations of Hox genes and it is thought, on the basis of the relative positions of expression cut-offs, that the boundaries correspond to the regions in which morphologically distinct rhombomere boundaries will later appear (Murphy *et al.*, 1989; Wilkinson *et al.*, 1989; Hunt *et al.*, 1991a; Murphy and Hill, 1991). Rhombomere-specific expression of particular Hox genes appears to be autonomous and maintained even following transplantation to an ectopic site (Guthrie *et al.*, 1992; Kuratani and Eichele, 1993). When neural crest cells emerge, they express a combination of genes closely related to their level of hindbrain origin (Frohman *et al.*, 1990; Hunt *et al.*, 1991c) and continue to do so after they have migrated into the branchial arches

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(Hunt *et al.*, 1991a). There is a constant relationship between the position of origin of a crest population within the neural plate and the branchial arch into which it migrates (Lumsden *et al.*, 1991), with the result that each branchial arch expresses a unique combination of Hox genes. Subsequently, the surface ectoderm of the branchial arches adopts the Hox expression pattern of the ectomesenchyme beneath it, consistent with a transfer of spatial information to the ectoderm (Hunt *et al.*, 1991c). These observations suggest that Hox genes may be part of the mechanism of spatial specification within the neural crest.

Several attempts have been made to alter the Hox code of the branchial region in mice by either deletion of genes or overexpression. If Hox genes are involved in specification of neural crest identity, it might be expected that such experiments would result in transformations in the identity of neural crest-derived structures consistent with any such alterations in Hox code. Experiments attempting to express single Hox genes beyond their normal expression domain have resulted in transformations of structures; however, in all cases paraxial mesenchyme was affected, neural crest derivatives not being subject this type of alteration (Kessel *et al.*, 1990; Lufkin *et al.*, 1992; Pollock *et al.*, 1992). In some experiments which produced loss of gene function, pattern deficits were observed in crest derivatives, rather than transformations of structures (Chisaka and Capecchi, 1991; Lufkin *et al.*, 1991; Chisaka *et al.*, 1992), although transformations of a restricted set of first arch derivatives have been observed in one case (Gendron-Maguire *et al.*, 1993; Rijli *et al.*, 1993). Hox genes are organized into subfamilies of genes, such that two, three, or four genes with very similar protein sequences are present (reviewed in McGinnis and Krumlauf, 1992). Members of such a related subfamily of genes are known as paralogues and are thought to have arisen as a result of the duplication of entire Hox clusters during evolution (Akam, 1989). Paralogous genes are expressed in identical combinations of rhombomeres and branchial arches at the early phases of facial development when regional identity is being established (Hunt *et al.*, 1991a). It may therefore be difficult to induce the transformation of structures by disruption of a single gene due to some functional redundancy among paralagous Hox genes; i.e., the remaining related genes, still expressed in their normal expression domains, may be able to maintain a mostly normal spatial specification of crest. In this scenario any defects observed would reflect the chronologically first developmental function of a Hox gene for which another gene could not substitute (Hunt and Krumlauf, 1991). In this context it is significant that where transformations have been seen, the paralogous group concerned contained only one other gene capable of compensating for lack of *Hox-A2* (Gendron-Maguire *et al.*, 1993; Rijli *et al.*, 1993).

In this paper we describe a complementary approach to investigate the role of Hox genes in neural crest patterning. Surgical manipulation of early chick embryos enables the experimenter to confront neural crest cells with inappropriate axial environments. Hox gene expression patterns and the subsequent morphogenetic fate of the crest cells involved can then be assessed to establish if the normal 1:1 correspondence is maintained or lost as a result. Recently the ability of the embryo to regulate for ablation of the neural crest has been reexamined with surprising results. Previous reports of "in-filling" migration, in which crest cells located immediately anterior and posterior to the region of ablation apparently migrate caudally and rostrally, respectively, to compensate for the deficit (McKee and Ferguson, 1984; Hall and Hörstadius, 1988), have been reassessed in the light of a report that mid- and hindbrain crests can be regenerated by the neuroepithelium remaining at the level of the ablation (Scherson *et al.*, 1993). DiI labeling revealed that "in-filling" migration has a negligible role in this regulation and that the neuroepithelium of the lateral wall of the mesencephalic and rhombencephalic neural tube is able (during a short developmental window) to regenerate crest cells which proceed to migrate along pathways normal for their axial level of origin (Scherson *et al.*, 1993). As those authors acknowledge, such a phenomenon constitutes a particular challenge to the specificity and plasticity of the branchial arch Hox code since the fate of the cells responsible has changed dramatically, from forming part of the mid- or hindbrain to giving rise to an as yet undefined range of crest derivatives. We have chosen to exploit this capacity of the neuroepithelium as the basis for exploring further the relationship among Hox code, branchial arch morphogenesis, and skeletal development. Using an ablation strategy in which a major part of the hindbrain is removed bilaterally, we have endeavored to answer a number of specific questions: (1) Is morphogenesis normal in the branchial region following crest ablation? (2) Is a normal branchial arch Hox code reestablished in those arches for which the contributing crest has been ablated and subsequently regenerated? and (3) What are the long-term morphogenetic consequences in terms of the (viscerocranial) skeleton, which is normally formed from ectomesenchyme derived from hindbrain crest?

The work presented here shows that branchial arch morphogenesis is normal after bilateral crest deletion, that a normal branchial Hox code is reestablished, and that normal skeletal development ensues.

MATERIALS AND METHODS

In Ovo Surgery

Fertilized White Leghorn eggs were obtained from commercial suppliers (J. K. Needle & Co., Goffs Oak, UK) and incubated at 37°C in a humidified incubator to Hamburger and Hamilton stages 8 and 9. The eggs were windowed, some albumin was removed, and several drops of αMEM (Gibco) containing 2.5 μg/ml fungizone (Gibco) were placed on the vitelline membrane over the embryo. Black "Pelikan" India ink was centrifuged briefly in a microfuge and the supernatant was diluted 1:9 with sterile phosphate-buffered saline (pH 7.3) and injected beneath the embryo to provide a contrasting background to facilitate the microsurgery. A slit was gently made in the vitelline membrane and crest deletions were performed bilaterally with a fine tungsten needle. The tissues removed are indicated in Fig. 1. After operation the windows were sealed with sellotape and incubated in a humidified incubator until required for further analysis. Video recordings were made of many of the freshly operated embryos, using a JVC (Model TK-870U) color video camera mounted on a Nikon stereo microscope, thereby providing a record of each individual operation.

Scanning Electron Microscopy

Embryos were harvested at 48 hr after operation, and the heart and adherent membranes were removed before overnight fixation in 3% glutaraldehyde in 0.1 *M* sodium cacodylate buffer, pH 7.4. The embryos were then dehydrated in ethanol, transferred to acetone, and critical point dried using carbon dioxide. The specimens were then sputter coated with gold/palladium and visualized at 15 kV in a Cambridge 90B stereoscan scanning electron microscope.

Whole Embryo Observation

Embryos which had increased in somite number after operation were dissected from the surrounding area vasculosa, mounted in MEM + 10% FCS on glass microscope slides, and viewed by Nomarski differential interference contrast microscopy.

Immunostaining

Embryos were fixed in 4% paraformaldehyde in PBS, ethanol dehydrated, and wax embedded. Sections were cut at 6 μm. Dewaxed, hydrated sections were washed successively in PBS and in PBS containing 0.05% Tween 20 (PBS-Tween) and then preincubated in αMEM containing 10% fetal calf serum (FCS) to block nonspecific staining. Slides were incubated with HNK-1 ascites diluted 1/200 in αMEM + 10% FCS for 2 hr at room temperature, followed by five washes in PBS-Tween for 30 min. Incubation with the secondary antibody, affinity-purified alkaline phosphatase-conjugated rabbit anti-mouse immunoglobulins diluted in αMEM + 10% FCS, was for 45 min at room temperature, followed by further washing (five times) in PBS-Tween for 30 min. Slides were then rinsed in alkaline phosphatase (AP) buffer (0.1 *M* Tris, pH 9.5, containing 0.1 *M* NaCl, 50 m*M* MgCl, 0.1% Tween −20) for 5 min, and the reaction was developed (usually over 30 min) in 10 ml of the AP buffer containing 0.1 *M* levamisole, 44 μl NBT, and 66 μl BCIP. Slides were then briefly rinsed in water to block the reaction and air-dried and then coverslips were mounted with glycerol, before photomicroscopy on a Zeiss Axiophot using Kodak T-Max 100ASA film.

In Situ Hybridization

The chicken *Hox-B4* probe used in this study was a 1.17-kb *Eco*RI fragment in PKS linearized with *Hin*dIII to give an antisense transcript with T3. The chicken *Hox-A3* probe was a 0.9-kb *Kpn*I/*Eco*RI fragment in PKS linearized with *Bam*HI to give an antisense transcript with T7.

In situ hybridization was basically as described in Wilkinson and Green (1990). Briefly, embryos were fixed with 4% paraformaldehyde in PBS, ethanol dehydrated, embedded in paraffin wax, and sectioned at 6 μm. Sections were dewaxed, postfixed in 4% paraformaldehyde, treated with 20 μg/ml proteinase K, refixed, and acetylated with acetic anhydride in triethanolamine buffer. After dehydration a transcribed riboprobe purified on a Sephadex G-50 drip column at a final activity of 1×10^5 cpm/μl in hybridization buffer was pipetted onto the slides and covered with a coverslip. Slides were hybridized at 55°C overnight in a humidified box, and then coverslips were removed in a solution of 5× SSC, 10 m*M* DTT (dithiothreitol) for 30 min. After a further 30 min in a change of the same solution, slides were washed at high stringency in 2× SSC, 10 m*M* dithiothreitol, 50% formamide for 30 min at 65°C. Slides were treated with 20 μg RNase A for 30 min at 37°C and then washed again at 65°C in a wash solution of the same composition. After dehydration, slides were dipped in Ilford K5 nuclear emulsion diluted 1:1 with 2% glycerol and exposed for 5 days at 4°C before being developed with Kodak D19 developer and counterstained with toluidine blue. Signal was visualized under bright field illumination and photographed using an Olympus BH2 photomicroscope on Kodak Ektachrome 160T color reversal film.

Whole Mount Cartilage and Bone Staining

Embryos were fixed and stained (Simons and Van Horn, 1971) in 80% ethanol, 20% acetic acid, 0.15 mg/ml

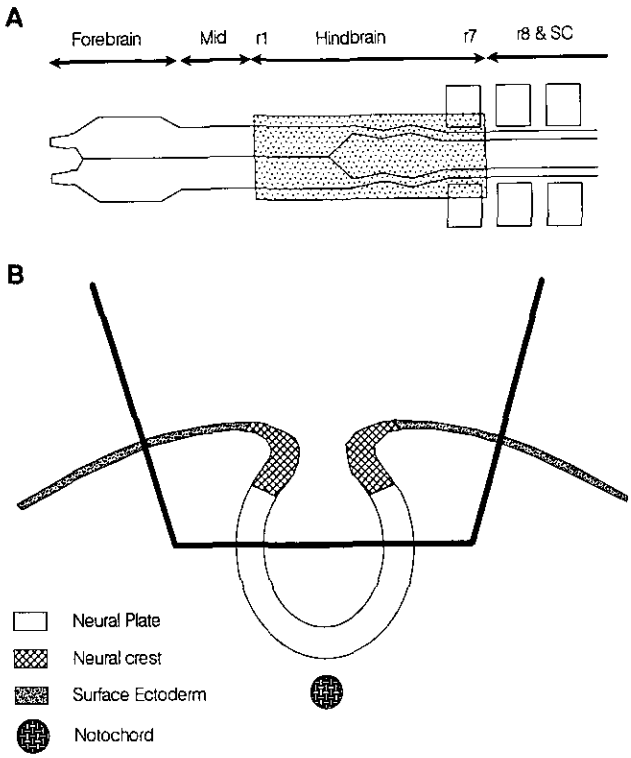


FIG. 1. Diagrammatic view of a transverse section through a stage 9 chick hindbrain, indicating regions removed during hindbrain crest deletion. (A) Rostrocaudal extent of neural crest deletions; (B) dorsoventral extent of neural crest deletions.

alcian blue, fully dehydrated through an ethanol series, stained with 0.1 mg/ml alizarine S in 0.5% KOH, cleared in 1% KOH, and then transferred into progressively higher concentrations of glycerol for photography on a Zeiss SV-11 stereo-microscope on Kodak Ektachrome 160T color reversal film.

RESULTS

Neural crest deletions were performed, using tungsten needles, on embryos *in ovo* between Hamburger and Hamilton stages 8+ and 9. Previous work involving unilateral mesencephalic crest deletions had failed to produce pattern defects, apparently due to the migration of neural crest from the contralateral side across the ventral midline (McKee and Ferguson, 1984); thus, in an attempt to produce a complete neural crest deletion for a particular axial level, ablations were routinely performed bilaterally. The tissue removed included regions of the surface ectoderm lateral of the neural folds, the neural folds themselves, and portions of the neural tube (Fig. 1). The rostral limit of the deletion was the rhombomere 1/midbrain boundary, identified by morphological criteria, while the caudal limit corresponded to either the rostral or caudal limit of somite 1. On the basis of the crest fate map constructed by Lumsden *et al.* (1991), this would correspond to the crest normally contributing most of the mesenchyme to the mandibular portion of arch I, the hyoid (second) and third arches. The inclusion of such extensive areas in the deletion and the timing to the stage when crest emigration is just beginning makes it unlikely that any early migrating crest cells are left after tissue removal. Control embryos were prepared in a way similar to operated embryos, but no further intervention was performed after the vitelline membrane was torn.

One hundred thirty-three embryos in total were operated upon, of which 17 (12.8%) died as a result of the operation, and 27 were harvested for Nomarski observation after they had increased their number of somites but before they had the opportunity to form branchial arches. Of the 103 embryos whose development was allowed to continue to the branchial arch stage, 15 (14.6%) suffered from a range of abnormalities, such as failure to establish a vascular supply, that were not specifically associated with craniofacial development. All of the remaining 78 (75.7%) reached branchial arch stage or beyond without abnormality (of the 78, 10 died between 48 hr postoperation and 10 days of development). This data is summarized in Table 1.

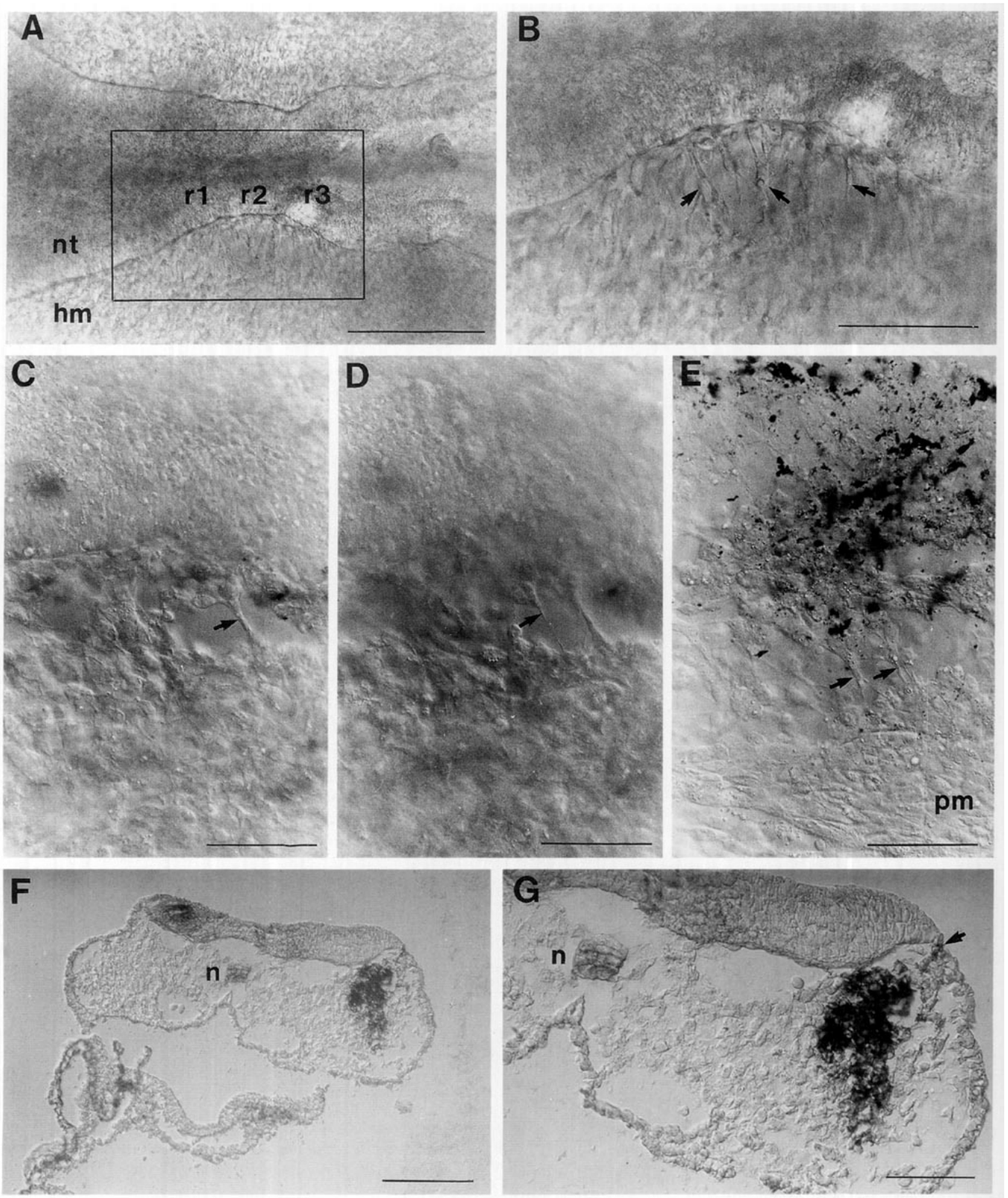
The Neuroepithelial Basis of Neural Crest Regeneration

We monitored the cellular events following ablation using Nomarski differential interference contrast microscopy (DIC) and immunocytochemistry in order to establish that regulation in this system is essentially equivalent to that reported by Scherson *et al.* (1993). Initially we examined the hindbrain region of embryos 4 hr after operation, embryos being selected for the normal-

TABLE 1
TOTAL NUMBERS OF EMBRYOS ANALYZED

Embryo category	Number
Total Operated	133
Died immediately after surgery	17 (12.7%)
Harvested for Nomarski observation	27
Total analyzed at branchial arch stage	103
Died of causes unrelated to craniofacial	15 (14.6%)
Normal morphology at branchial arch	78 (75.7%)
Died before 10 days of development	10 (9.7%)

Note. For the first three categories, the first percentage is of the total number of embryos operated. The bottom three percentages are of the 103 embryos which survived at least to the stage at which branchial arches are obvious. The 10 which died before 10 days of development were intended for whole mount skeletal preparations, but having survived normally for 48 hr after operation died at some stage between this point and 10 days.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ity of their continued development on the basis of overall morphology and the number of somite pairs increasing by 3–4.

On focusing to a level subjacent to the dorsal and dorsolateral ectoderm, a particular cell phenotype and arrangement was routinely seen in all operated embryos. At the level of the original deletion, and even prior to closure of the neural tube within this region, a number of elongated cells with a mesenchymal phenotype and with their long axes at right angles to the embryonic axis were seen emanating from the edges of the neuroepithelium on both sides of the ablation, as shown in Fig. 2. These elongated cells spanned, or appeared to be in the process of spanning, the extracellular space created by the ablation 4 hr earlier. Figure 2A shows an overall view of the rostral hindbrain at this stage, while Fig. 2B is a detail of the same field at the r2 level. Cells with elongated processes perpendicular to the long axis of the neural tube are indicated by arrows. Optical sectioning of an embryo at the r2 level revealed a contrast between dorsal cells with perpendicularly orientated processes, indicated by arrows in Figs. 2C and 2D, and more ventral levels in which cells with longitudinal orientation and irregular shape were located (pm), as shown in Fig. 2E. The longitudinally oriented cells in ventral sections (pm) are tentatively identified as paraxial mesoderm. The elongated cells located more dorsally were interpreted as evidence of neural crest regeneration from the cut edge of the neuroepithelium, their mesenchymal morphology reflecting a deepithelialization of cells at that edge which, in a normal intact embryo, would have remained a part of the neuroepithelial wall of the hindbrain neural tube.

To confirm this interpretation, embryos at 4 and 18 hr after operation were prepared for immunostaining using the HNK-1 monoclonal antibody which recognizes a carbohydrate prosthetic group shared by a number of avian cell adhesion proteins but which, at this stage of development, is specifically expressed by emigrating neural crest cells (Tucker *et al.*, 1984; Vincent and Thiery, 1984). Transverse sections through embryos at levels adjacent to and within the region of the deletion revealed no HNK-1-positive cells at 4 hr after operation levels. At levels rostral and caudal to the deletion, immunopositive cells were found at locations well advanced on crest migration pathways as previously defined (Tucker *et al.*, 1984; Vincent and Thiery, 1984; Lumsden *et al.*, 1991) (data not shown). A section of an embryo examined 18 hr after operation is shown in Fig. 2F, revealing HNK-1-positive cells in a stream emanating from the repairing neural tube and mostly situated immediately subjacent to the dorsal and dorsolateral ectoderm. The relative position of this section rostral to the otocyst suggests that it corresponds to the r2 level. A detail of this section is shown in Fig. 2G, in which a HNK-1-positive cell is in contact with the lateral edge of the neuroepithelium, indicated by an arrow. Given their location, appearance, and HNK-1 immunopositivity, and the evidence of postoperative replacement of ablated neural crest, these cells have been interpreted as neural crest in character and we have concluded that the system is regulating in a fashion similar to that described by Scherson *et al.* (1993).

Branchial Arch Morphology after Crest Ablation

The surface morphology of six embryos 48 hr after extensive crest removal, together with that of four controls, was investigated by SEM, and a selection of the results is shown in Fig. 3. In control embryos, examined 48 hr postoperatively, four pairs of branchial arches can be distinguished, the mandibular (I), hyoid (II), third (III), and fourth (IV) arches (Fig. 3A), displaying various degrees of fusion and depth of interarch branchial cleft. Epithelial protuberances (ep) at the proximal aspect of first and second branchial clefts were regularly observed (Fig. 3B) which were interpreted as epibranchial placodes (D'Amico-Martel and Noden, 1983; Le Douarin *et al.*, 1986); these were found in all control embryos examined but their relative prominence varied and this may reflect subtle variations in developmental age.

FIG. 2. Cellular properties associated with the cut edge of the neuroepithelium. (A–F) Nomarski images of chick embryos 4 hr after bilateral rhombencephalic crest ablation; (F and G) Transverse sections of an embryo 18 hr after bilateral rhombencephalic crest deletion, stained with HNK-1. (A) Dorsal view of the rostral rhombencephalon of an embryo from which neural crest was bilaterally deleted at 7 somites and harvested at 11 somites. Rostral is to the left. Bar, 10 μm. (B) Enlargement of region centered on r2 indicated in A. Arrows indicate cells associated with the edge of the neuroepithelium with transversely orientated cell processes. Bar, 5 μm. (C) Dorsal view of the edge of the neuroepithelium of an embryo operated on at 7 somites and harvested at 11 somites. A transversely orientated cell adjacent to r2 is indicated by the arrow. Bar, 5 μm. (D) More ventral view of the same field shown in D, with an additional orientated cell process indicated. Bar, 5 μm. (E) More ventral view of the same field shown in D. Note the contrast between the lateral orientation between the cells indicated by arrows, assumed to be neural crest in origin, and the cells of the paraxial mesoderm, with a longitudinal or more random orientation. The dark staining is residual India ink used to provide a dark background when performing neural crest deletions. Bar, 5 μm. (F) Transverse section through rhombomere 2 of an embryo from which neural crest was deleted at 11 somites and analyzed at 22 somites. Bar, 10 μm. (G) High-power view of the section shown in F. The presence of a HNK-1-positive cell at the interface between the neural plate and the surface ectoderm is indicated by an arrow. Bar, 5 μm. nt, neural tube; hm, head mesenchyme; pm, paraxial mesenchyme; n, notochord.

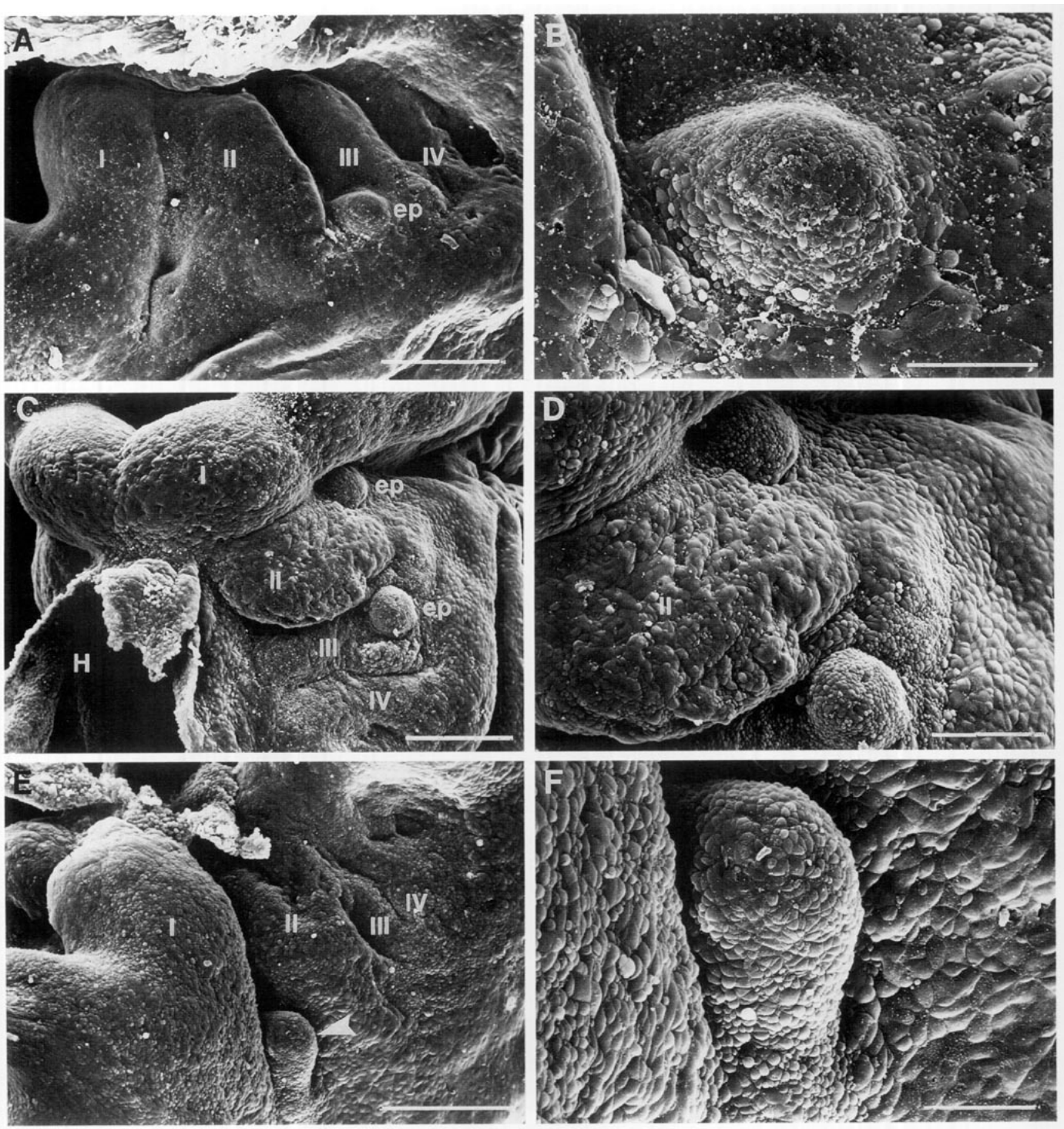


FIG. 3. External facial morphology of chick embryos 48 hr after bilateral rhombencephalic crest deletion. (A) Lateral view of the branchial arch region of a control stage 18 embryo, showing the normal appearance of arches I–IV. Rostral is to the left, ventral is uppermost. Note the epithelial protuberance associated with the dorsal rostral part of arch III. Bar, 200 μm. (B) Detail of the epithelial protuberance illustrated in A. Bar, 50 μm. (C) Oblique frontal view of the lefthand side of the branchial region of an stage 17 embryo 48 hr after operation. Rostral is toward the top. The heart primordium has been removed, and the cavity of the outflow tract is indicated. All four branchial arches are relatively normal in size and position, although there are protuberances associated with the dorsal rostral parts of the second and third arches. Bar, 200 μm. (D) Detail of the protuberances indicated illustrated in C. Bar, 100 μm. (E) Right lateral view of the branchial region of the most severely affected embryo. Rostral is toward the left, ventral is toward the top. Note the reduced branchial arches and the protuberance associated with arch I. Bar, 200 μm. (F) Detail of the protuberance shown in E. Bar, 50 μm. ep, epithelial protuberance; H, cavity of heart outflow tract.

In each of the six operated embryos studied, there was a largely normal branchial arch morphology, typically showing normal sized and shaped mandibular and hyoid arches with perhaps a slight reduction in the size of the third and fourth arches (Figs. 3C–3F), although this might simply result from a slight retardation in arch morphogenesis (see following). The range of morphologies observed included a slightly greater variation in the size of the epibranchial placodes than that seen in the control embryos. In the most extreme case a protuberant structure was observed at the proximal part of the first branchial cleft (Figs. 3E and 3F). This embryo was exceptional; however, the remaining operated embryos had a morphology similar to that illustrated in Figs. 3C and 3D.

In summary, at the level of surface morphology the branchial region of most operated embryos appears largely normal, despite the removal of the tissue that would provide the underlying mesenchyme in normal development. Even in more severely affected embryos there was clearly extensive mesenchyme present in the branchial region, since it is unlikely that the surface morphology observed could exist without an underlying core of mesenchyme. We conclude that the regulative capacity of the system allows the reestablishment of an ectomesenchymal population capable of building a morphologically normal set of branchial arches.

Hox Expression Domains in Operated Embryos

Having established that the external morphology of the branchial arches is grossly normal in the majority of embryos from which the hindbrain neural crest has been removed, we investigated the pattern of Hox gene expression in the branchial apparatus of four similar embryos in which the rhombencephalic crest had been bilaterally removed. Two nonparalogous "indicator" genes were selected on the basis of their known expression limits in mouse: chicken *Hox-A3* (formerly chicken *Hox-1.5*) and chicken *Hox-B4* (formerly chicken *Hox-2.6*). Murine *Hox-A3* is known to display rostral expression limits at the rhombomere 4/rhombomere 5 boundary in the hindbrain and to be expressed in the third branchial arch and more caudal areas of mesenchyme (Hunt *et al.*, 1991a). *Hox-B4* is expressed up to the rhombomere 6/rhombomere 7 boundary in the hindbrain and the fourth branchial arch (Wilkinson *et al.*, 1989; Hunt *et al.*, 1991c).

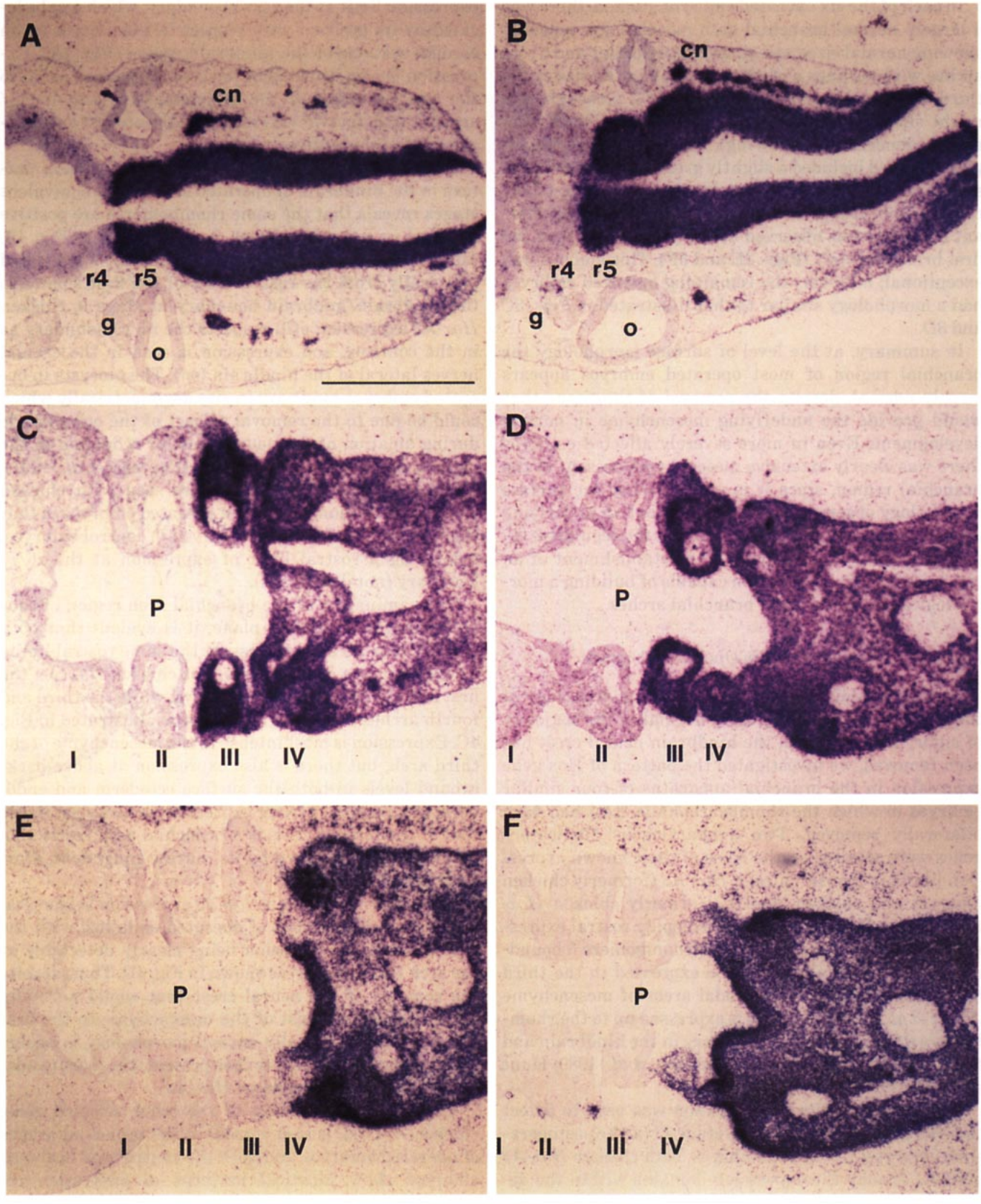
Radioactive *in situ* hybridization was used to detect the endogenous transcripts in stage 17 (48 hr postoperative) embryos. In control embryos, both chicken *Hox-A3* and *-B4* display the expression domains within the developing hindbrain predicted on the basis of their murine counterparts. Thus, chicken *Hox-A3* shows a rostral expression limit at the r4/r5 boundary, which can be located by its position with respect to the otocyst (o) in coronal sections of the hindbrain region (Fig. 4A). Expression is also detected in the cranial nerves caudal to the otocyst although the trigeminal (v) and facial/acoustic ganglia (vii/viii) rostral of the otocyst are characteristically negative.

Examination of the chicken *Hox-A3* expression pattern in the hindbrain of operated embryos at equivalent stages reveals that the same rhombomeres are positive as those in control embryos, as illustrated in Fig. 4B. Thus, in embryos in which the neural crest was deleted bilaterally from the rostral edge of the first somite to the hindbrain/midbrain boundary at stage 9, chicken *Hox-A3* expression still respects the r4/r5 boundary as in the controls, and expression is seen in the cranial nerves lateral of the hindbrain (cn). The otocysts in operated embryos may vary in size asymmetrically which could be due to the removal of part of the otic placode during ablation of the neural crest or reduction in the amount of *int-2* secreting tissue (Represa *et al.*, 1991). Essentially equivalent results are seen with chicken *Hox-B4* expression domains in operated embryos in that they are identical to those observed in control embryos, displaying a rostral limit of expression at the r6/r7 boundary (not illustrated).

On examination of the branchial arch region of control embryos in coronal plane, it is evident that both chicken *Hox-A3* and *-B4* respect the same rostral limits as their murine counterparts. For chicken *Hox-A3*, the first and second arches are negative, while the third and fourth arches are strongly positive as illustrated in Fig. 4C. Expression is most intense in the mesenchyme of the third arch, but there is also expression at above-background levels in both the surface ectoderm and endoderm of this arch. Strong expression is also seen in the roof of the foregut. This is a branchial arch restriction identical to that shown by its murine equivalent, *Hox-1.5* (Hunt *et al.*, 1991a).

The pattern of chicken *Hox-A3* expression is identical in the branchial arches of operated embryos, with the rostral limit of expression being clearly detectable at the arch III boundary, as shown in Fig. 4D. Thus, despite the removal of the neural crest that would normally have contributed most of the mesenchyme to the first three arches, a basically normal morphology is apparent, with four branchial arches present, the more caudal two both expressing chicken *Hox-A3*.

The expression domain of the other selected gene, chicken *Hox-B4*, is also normal in the branchial arches of operated embryos and both the control and operated embryos show identical patterns of expression restricted to the fourth arch and caudal regions, as shown in Figs. 4E and 4F. The caudal extent of the original de-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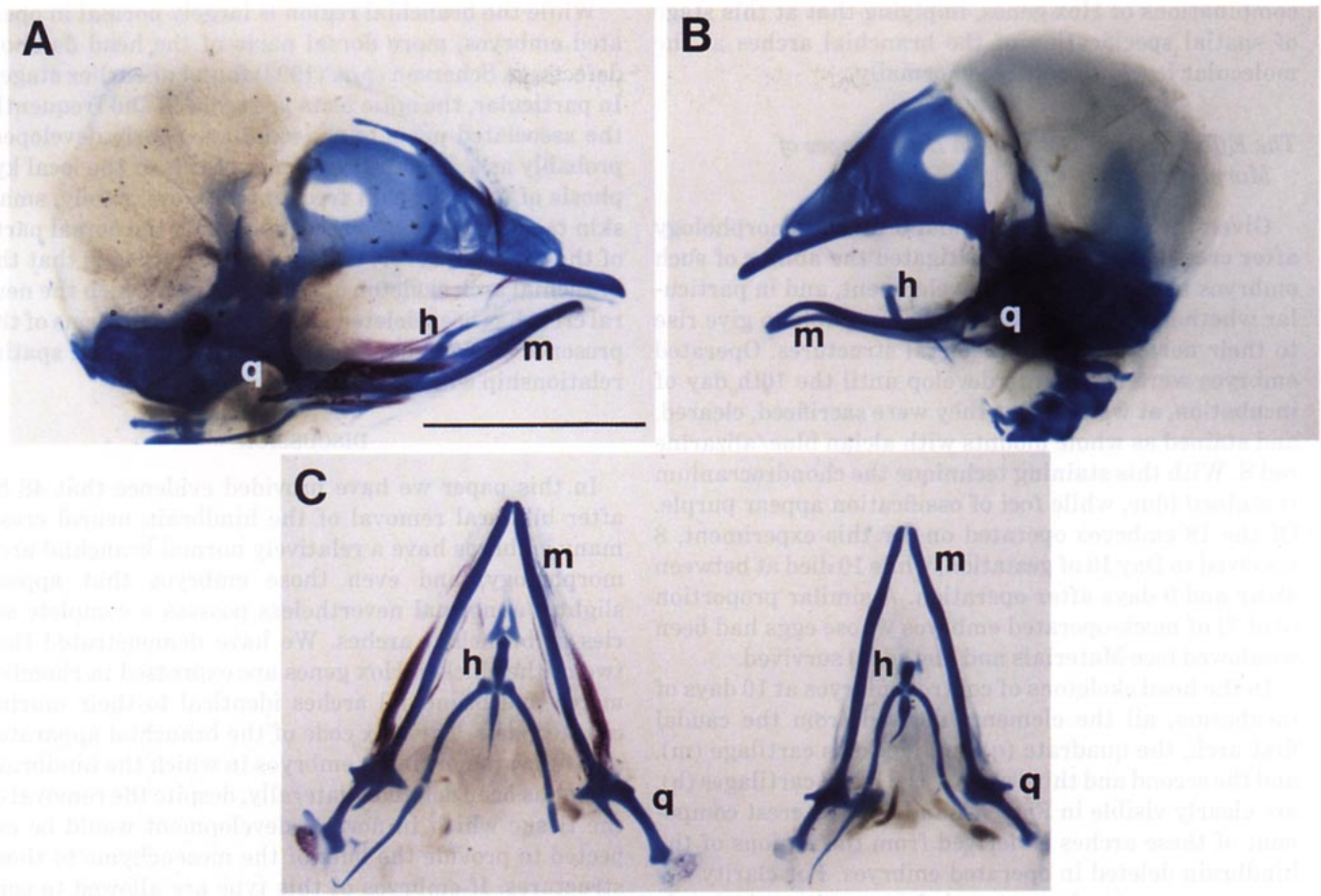

FIG. 5. Skull morphology of embryos 10 days after operation. Embryos were cleared with potassium hydroxide and stained in whole mount with alcian blue and durazole red. The eyes have been removed for clarity. Bar, 0.5 cm. (A) Control embryo at stage 37, lateral view. Note location and shape of the quadrate, Meckel's cartilage, and the hyoid arch. (B) Operated embryo at stage 35. (C) Ventral aspect of isolated jaws of the two embryos. Control to the left and operated to the right. Note normality in shape and location of skeletal elements in the operated embryo. q, quadrate; m, Meckel's cartilage; h, hyoid cartilage.

letion would not have included rhombomere 7, the normal limit of chicken *Hox-B4* expression; nevertheless, a deletion of crest immediately rostrally might have been expected to perturb the migration pathways of a more caudal crest. Figure 4F provides no evidence for expression in the third arch, as might be expected if crest cells from r7 had maintained their original expression patterns and migrated there.

In summary, 48 hr after bilateral rhombencephalic crest deletion embryos have a branchial arch apparatus which not only closely resembles that of control embryos at a morphological level, but expresses the same

FIG. 4. Hox expression in the branchial region of neural crest deleted and normal chick embryos. In all cases bright-field views of toluidine blue-stained sections hybridized with radioactive *in situ* probes are shown. A–D are hybridized with *Hox-A3*, and E and F are hybridized with *Hox-B4*. The deposits of silver grains over regions of expression are so intense that they have stained strongly with toluidine blue. All sections shown at same magnification. Bar, 500 μm. (A) Coronal section of the hindbrain of a stage 18 normal chick embryo. Rostral is to the left. Note expression throughout caudal hindbrain with a rostral limit at the r4/r5 boundary and expression in cranial nerves. (B) Equivalent section of a crest-deleted embryo at the same stage. Expression within the hindbrain is normal. (C) More ventral section in the same series as A, passing through the branchial arches. Note intense *Hox-A3* expression in the third and more caudal branchial arches. (D) Equivalent section of the same operated embryo shown in B. The expression pattern within the branchial arches is identical to that of the control. (E) Near adjacent section to C of the control embryo, hybridized with *Hox-B4*. Note expression in arch IV and more caudal regions of the embryo. (F) Near adjacent section to that shown in D, hybridized with *Hox-B4*. Note normal expression pattern in branchial arch IV and more caudal regions, with no expression in any more rostral parts of the body. o, otocyst; r4, r5, rhombomeres 4 and 5; cn, cranial nerves; P, pharynx; g, vii/viii ganglion complex.

combinations of Hox genes, implying that at this stage of spatial specification of the branchial arches at the molecular level has occurred normally.

The Effect of Crest Deletion on Later Stages of Morphogenesis

Given the surprisingly normal branchial morphology after crest deletion, we investigated the ability of such embryos to continue their development, and in particular whether the branchial arches were able to give rise to their normal range of skeletal structures. Operated embryos were allowed to develop until the 10th day of incubation, at which point they were sacrificed, cleared, and stained as whole mounts with alcian blue/alizarine red S. With this staining technique the chondrocranium is stained blue, while foci of ossification appear purple. Of the 18 embryos operated on for this experiment, 8 survived to Day 10 of gestation, while 10 died at between 48 hr and 9 days after operation. A similar proportion (3 of 7) of mock-operated embryos whose eggs had been windowed (see Materials and Methods) survived.

In the head skeletons of control embryos at 10 days of incubation, all the elements derived from the caudal first arch, the quadrate (q) and Meckel's cartilage (m), and the second and third arches, the hyoid cartilages (h), are clearly visible in Figs. 5A and 5C. The crest component of these arches is derived from the regions of the hindbrain deleted in operated embryos. For clarity the eyes were routinely removed from embryonic heads prior to photography. Head skeletons of embryos, from which the rhombencephalic crest rostral to the first somite was bilaterally removed, contain all the same elements and it is important to note that Meckel's cartilage and the quadrate, derived from the first branchial arch, are of normal relative size and position with respect to the other parts of the skull (Figs. 5B and 5C). Note that the embryos illustrated in Figs. 5A and 5B display a slight disparity in size reflecting the more advanced developmental stage of the control embryo after equivalent postoperative periods. We interpret this as evidence of slight retardation of development in the operated embryos as a consequence of operative trauma and a recovery period.

From a ventral perspective, the normality of jaw pattern is even more evident. The characteristic V-shaped morphology of Meckel's cartilage (m), derived from the first arch, and the hyoid apparatus (h), derived from the second and third arches, is seen clearly in both control and operated embryos (Fig. 5C). The slight disparity in size again reflects the stage of the operated embryo, stage 35 as opposed to stage 37 in the control, and both morphologies are normal for the stages of development achieved (Vorster, 1989).

While the branchial region is largely normal in operated embryos, more dorsal parts of the head do show defects, as Scherson *et al.* (1993) found at earlier stages. In particular, the optic tecta are reduced and frequently the associated parts of the skull are poorly developed, probably as a direct consequence of this or the local kyphosis of the neck that frequently occurs; rarely, small skin tags may be found associated with the dorsal parts of the neck. However, the significant finding is that the branchial arch skeleton of embryos from which the neural crest has been deleted appears normal in terms of the presence of elements and their shape, size, and spatial relationship with other skull components.

DISCUSSION

In this paper we have provided evidence that 48 hr after bilateral removal of the hindbrain neural crest, many embryos have a relatively normal branchial arch morphology, and even those embryos that appear slightly abnormal nevertheless possess a complete series of branchial arches. We have demonstrated that two of the chicken Hox genes are expressed in rhombomeres and branchial arches identical to their murine counterparts. The Hox code of the branchial apparatus also appears normal in embryos in which the hindbrain crest has been deleted bilaterally, despite the removal of the tissue which in normal development would be expected to provide the bulk of the mesenchyme to these structures. If embryos of this type are allowed to continue their development, they are able to produce a branchial skeleton appropriate in appearance and position with respect to the rest of the skull, which suggests that they are able to execute extensive parts of their developmental program normally. Analysis of embryos 18 hr after crest deletion reveals a number of orientated cells associated with the cut edges of the neuroepithelium, whose location, morphology, and HNK-1 immunopositivity suggest that they are regenerated neural crest cells, thus confirming the reported ability of the neural tube to regulate following crest ablation (Scherson *et al.*, 1993).

Crest Deletion and the Branchial Arch Hox Code

These experiments were conducted to test the 1:1 correspondence between the combinatorial expression of the 3′ Hox genes of the "A" and "B" clusters and the specification of form and tissue patterning in the branchial arches. Our attention has focused on the skeletogenic fate of the arch mesenchyme but clearly it will be important in future work to examine patterns of innervation and organization of the cranial ganglia. The single most important fact to emerge from the data is that normal branchial arch morphogenesis ensues after

ablation of most of the rhombencephalic crest and that in these tissues, the 1:1 correspondence is maintained as judged by the expression domains of the two "indicator" genes used (*Hox-A3* and *-B4*). Irrespective of the origin of the cells that provides the arch mesenchyme, the 1:1 correspondence is clearly maintained or, at least, reestablished, during regeneration. Therefore the hypothesis that the combinatorial expression of these genes and their paralogues is causally linked with the morphogenetic fate of the branchial arches (Hunt *et al.*, 1991b) is upheld. The nature of that causal relationship and of the various levels of genetic control that must exist between Hox code and phenotype in different regions of the developing head remains to be defined. This conclusion, and the manner in which the relatively normal branchial arch development is reestablished, has several important implications for the study of neural crest development generally.

The Source and Potential of Branchial Arch Mesenchyme following Neural Crest Ablation

Scherson *et al.* (1993) showed by DiI labeling that after unilateral and bilateral deletion, neural crest cells originate from the neuroepithelium and migrate normally and state that normal branchial arch morphology was normal 36 hr after bilateral crest ablation. We have complemented and extended their findings, and in particular we have investigated the ability of the regenerated crest to participate in subsequent development.

On the basis of Scherson *et al.* (1993) and our analysis of crest migration by DIC microscopy and HNK-1 staining, it appears that the neuroepithelium is the source of the crest cells arising after crest ablation in our system. An alternative explanation could be that the normal neural crest is not being removed in its entirety, and we are describing the ability of the remaining cells from the same axial level to regulate in number and thus compensate for the deletion. Given the recent claim (Couly *et al.*, 1993) that cranial crest cells, at least those destined to form the bones of the cranial vault, commence migration at early stage 8, this is a possibility. However, this explanation seems unlikely for a number of reasons. First, since the deletions are performed at the time when crest cells are leaving the neuroepithelium, and the tissues removed include not only surface ectoderm and neural tube, but also associated regions of dorsal mesenchyme, ablated tissue will include even the earliest migrating crest cells. Second, like Scherson *et al.* (1993) we found regenerating crest cells migrating into the branchial arch region and, in this paper, confirmed their crest identity by HNK-1 immunostaining. Third, our ablated embryos were developmentally delayed which is unlikely to be the case if skeletogenic cells are already "in place" but is compatible with the conclusion that crest cells with skeletogenic potential have to be regenerated and then migrate into the arches.

It is known that neural crest is able to cross the ventral midline, and thus it could be argued that in the experiments assessing the developmental potential of regenerated crest carried out by Scherson *et al.* (1993), which involved unilateral ablation, normal development could have been maintained by the contralateral crest that was not ablated. In fact, in the smaller number of experiments in which they used bilateral ablation, equivalent results were obtained, and they concluded that crossover by crest was not a problem. Nevertheless, we felt it necessary to use bilateral crest deletion throughout our work, and we have shown that, under these rigorous conditions, regenerated crest alone is indeed capable of producing normally patterned structures.

In recent experiments involving deletion of rhombomere 4 in its entirety in the transverse plane, there was no evidence of HNK-1-positive cells rostral and ventral of the otocyst, the normal location of presumptive arch 2 crest, 14 hr after operation (Kuratani and Eichele, 1993). However, by 48 hr after operation a normal arch 2 morphology is shown (Kuratani and Eichele, 1993); Fig. 3C), suggesting that crest regeneration eventually occurs. It may be that the longitudinal deletions described in our work permit more rapid regeneration of HNK-1-positive cells than transverse deletions, where a regeneration to rejoin the severed parts of neuroepithelium must also occur. The greater time required for regeneration may explain the absence of HNK-1-positive cells 18 hr after a complete excision of r4 (Kuratani and Eichele, 1993).

When the neural crest caudal to the otocyst is deleted, defects in heart outflow tract septation (Bockman *et al.*, 1987; Nishibatake *et al.*, 1987) and thymic development (Kuratani and Bockman, 1990) result. The fact that in this case crest deletion did result in altered morphology could be related to the timing of neural crest migration into the heart; crest ablation and consequent regeneration may interfere with a precise timetable of migration at this level along the axis. Alternatively the crest of the cardiac region may differ in its intrinsic regenerative ability from more rostral crest; hence, patterning deficits are caused on its removal.

If, as we have suggested, cut edges of neuroepithelium have the ability to generate neural crest cells in response to wounding, this has implications for the interpretation of the many previous experiments involving grafts of pieces of neuroepithelium or fate mapping by ablation; in either case, unrecognized regenerative ability of the neural crest adds a previously unacknowledged level of complexity.

Cell Lineage Considerations

Our conclusion that the neural crest as an embryonic cell population can be regenerated, at least at hindbrain level, has implications for the issue of cell lineage within both the regenerated crest itself and the neuroepithelium. What range of differentiative potential is displayed by the crest population which has been formed in response to ablation? Is it initially pluripotential in the sense that crest has been previously reported to be (Bronner-Fraser and Fraser, 1988)? In this series of experiments we have examined only the differentiation of an ectomesenchymal lineage. Are the other crest lineages also represented and are they represented in proportions faithfully reflecting those found in the crest that would have formed normally in an intact embryo? In other words are the normal sensory, parasympathetic, glial, and melanogenic derivatives also formed in the postoperative embryo?

Additionally, patterning in the rostrocaudal axis of the neuroepithelium appears to be independent of the dorsoventral axis. After bilateral crest excision a normally patterned sequence of skeletal structures is formed, prefigured by the structures' associated patterns of Hox expression, implying that rostrocaudal positional values are sufficiently stable to be maintained in regenerated crest. We show that rather than a "reestablishment" of an axial level-specific Hox code, there is simply a "maintenance" of Hox gene expression characteristic of the neural tube at that level, i.e., as there is during normal hindbrain crest ontogeny. The excision removes the dorsal third of the rhombencephalic neural tube, so clearly neuroepithelial cells whose fate would have been to become part of the hindbrain can, under these experimental circumstances, acquire a very different set of dorsoventral fates. Does this represent, in some extreme form, the retention by the neuroepithelium/neural plate of a capacity for self-renewal through a stem cell mechanism, as recently suggested for the neural crest as a whole (Anderson, 1989)? Brain defects were noted and we cannot tell if these are the result of trauma or simply reflect the exhaustion of a neuropoeitic stem cell capacity. However, it is clear that whereas regional specificity along the rostrocaudal axis, mediated at some regulatory level by the Hox genes, is established prior to the period of experimental perturbation used here, the dorsoventral regional specification appears labile during this period. Thus, during regeneration cells along the dorsoventral axis of the hindbrain neural tube have switched fates from neuroepithelial/hindbrain to neural crest, or at least its ectomesenchymal lineage, but retain their regional specification along the rostrocaudal axis. As a corollary, it can be concluded that regional specification along the rostrocaudal axis precedes that along the dorsoventral axis in normal development.

Conclusions

We have demonstrated that regenerating neural crest cells retain a normal branchial Hox code 48 hr after deletion. Not only does the crest retain axial level specific information from the neural tube, but it also goes on to produce a normal skeletal morphology. This suggests that the Hox genes are a necessary part of the developmental program which produces that morphology.

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